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Effect of treatment with retinyl palmitate, progesterone, oestradiol and tamoxifen on secretion of a protein similar to retinol-binding protein during uterine gland development in neonatal pigs

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Previous work has demonstrated that uterine secretion of a protein with M_r 20 000 and pI 5.5 increases during neonatal endometrial gland development. Uterine tissue was collected from a 60-day-old gilt and cultured in 0.1 × leucine minimum essential medium (MEM) plus 50 μCi [^{3}H]leucine to determine whether this protein is related to retinol-binding protein (RBP). Conditioned medium was immunoprecipitated using anti-human RBP antiserum. A radioactive protein with M_r 20 000 and pI 5.5 was specifically immunoprecipitated from the conditioned medium. Uteri from neonatal gilts were collected at birth (day 0) and on days 3, 6, 9 and 12, cultured, and secreted proteins were immunoprecipitated as described above to determine whether secretion of immunoreactive RBP increased coincident with initiation of endometrial gland development. Immunoprecipitation demonstrated that the secretion of immunoreactive RBP increased by day 3. Finally, gilts were treated each day with corn oil, retinyl palmitate (10 000 iu day^{-1}), progesterone (20 mg day^{-1}), oestradiol (100 μg day^{-1}) or tamoxifen (1 mg day^{-1} or 0.1 mg day^{-1}) for 14 days beginning at birth to determine the effects of these treatments on endometrial gland development and uterine secretion of immunoreactive RBP. On day 14, gilts were killed and uteri collected. Uterine tissue samples were prepared for histology (to evaluate uterine development using morphometry) and for culture in 0.1 × methionine MEM plus 25 μCi [^{35}S]methionine (to evaluate uterine protein synthesis). Secretion of immunoreactive RBP was evaluated by immunoprecipitation. Retinyl palmitate increased ($P < 0.05$) glandular epithelial area without altering other uterine components or secretion of uterine immunoreactive RBP. Progesterone decreased ($P < 0.05$) secretion of uterine immunoreactive RBP but did not affect uterine histological measurements. Treatment with oestradiol and 1 mg tamoxifen day^{-1} stimulated ($P < 0.01$) secretion of uterine immunoreactive RBP and increased all the uterine components measured ($P < 0.01$). These results are consistent with a role for RBP and retinol in the development of the uterus during the neonatal period.

Introduction

The size of litters in pigs is influenced by ovulation rate, fertilization rate, embryonic mortality and uterine capacity (Christenson *et al.*, 1987). The extent and efficiency of uterine development during prepuberty in gilts can be expected to influence uterine capacity during pregnancy.

At birth, the uterine wall consists of a perimetrial epithelial layer, developing myometrial layers and an endometrial layer made up of stromal cells and luminal epithelial cells. No endometrial glands are present. However, within one week, endometrial glands begin to differentiate and continue to develop for the next 2–3 months (Hadek and Getty, 1959; Bal and Getty, 1970).

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Recent studies in pigs indicate that uterine secretion of several proteins change during the period of endometrial gland development (Christenson *et al.*, 1992; Spencer *et al.*, 1992). One of these proteins (M_r 20 000, pI 5.5), which is similar to porcine retinol-binding protein (RBP; Clawitter *et al.*, 1990; Harney *et al.*, 1990; Trout *et al.*, 1991), increases during the period of uterine gland development. Retinol is known to influence differentiation in various tissues, including several types of epithelium (DeLuca, 1991) through the regulation of genes such as the genes encoding keratin (Stellmach *et al.*, 1991), tissue plasminogen activator (Rickles *et al.*, 1989), laminin (Vasios *et al.*, 1989), matrix G1a protein (Cancela and Price, 1992) and transglutaminase (Yuspa *et al.*, 1982). Age-related changes in neonatal uterine secretion of RBP suggest that retinol may be involved in the process of uterine gland development.

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If an association between the secretion of RBP and endometrial morphogenesis can be demonstrated, factors affecting RBP secretion may help elucidate the role of RBP in endometrial morphogenesis. The adult porcine endometrium secretes RBP in response to progesterone (Adams *et al.*, 1981) and possibly oestrogen (Trout *et al.*, 1992). Chronic progestin exposure from birth to day 13 inhibited endometrial gland development in neonatal sheep (Bartol *et al.*, 1988). In addition, retinol is required for efficient hepatic secretion of RBP (Goodman, 1979; Ronne *et al.*, 1983). Therefore, progesterone and retinol could be used to investigate relationships between uterine wall development and RBP secretion in neonatal gilts. Oestradiol has been shown to stimulate uterine development in neonatal gilts (Spencer *et al.*, 1992). Tamoxifen increases uterine mass in pigs (Lin and Buttle, 1991) but inhibits gland development in rats (Branham *et al.*, 1985a). Therefore, oestradiol and tamoxifen could also be used in testing the association between uterine development and RBP secretion in neonatal gilts.

The objectives of this study were to: (1) determine whether the M_r 20 000, pI 5.5 protein crossreacts with antiserum to human RBP; (2) characterize the secretion of this protein by the uterus during the first 2 weeks of life in gilts and (3) determine whether treatment with retinyl palmitate, progesterone, oestradiol or tamoxifen influences uterine development and protein secretion (including putative RBP).

Materials and Methods

Experiment 1

The uterus of a 60-day-old crossbred gilt ($\frac{1}{4}$ Yorkshire, $\frac{1}{4}$ Large White, $\frac{1}{4}$ Chester White and $\frac{1}{4}$ Landrace) was collected at slaughter and 500 mg of tissue was cultured in 15 ml 0.1 × leucine minimum essential medium (MEM; Sigma Chemical Co., St Louis, MO) together with 50 μCi [^{3}H]leucine (Amersham Corp., Arlington Heights, IL). The tissue was cultured for 24 h at 37°C in an atmosphere of 50% N_2:45% O_2: 5% CO_2 (Vallet and Christenson, 1993) and conditioned medium was collected by centrifugation (10 min, 2500 ***g***, 4°C).

Duplicate samples (3 ml) of conditioned medium were incubated overnight sequentially with 50 μl anti-human RBP antiserum (Dako, Carpinteria, CA) or normal rabbit serum, followed by 100 μl 10% (w/v) protein A linked to Sepharose CL-4B previously equilibrated in 50 mmol Tris l^{-1}, 0.3 mol NaCl l^{-1}, 1 mmol EDTA l^{-1}, 2% (v/v) Triton X-100 and 0.02% (w/v) sodium azide (Buffer A). Samples were centrifuged (10 min, 2500 ***g***, 4°C), the supernatant discarded and the pellet washed five times with Buffer A. One hundred microlitres of 5 mmol K_2CO_3 l^{-1}, 2% Triton X-100 (v/v), 9.16 mol urea l^{-1}, 0.5% (w/v) dithiothreitol were then added and samples subjected to two-dimensional PAGE. Gels were then prepared for fluorography (28-day exposure; Roberts *et al.*, 1984).

Experiment 2

Twenty crossbred neonatal gilts were randomly assigned at birth (day 0) to be killed on day 0, 3, 6, 9 or 12 of age ($n = 4$ per day). Gilts were stunned with a captive bolt, exsanguinated and the uterus collected aseptically. Uteri were weighed and each uterus with a mass of less than 400 mg was cut into small pieces using a scalpel and cultured. Each uterus weighing more than 400 mg was evenly distributed between two culture plates. Tissues were cultured as previously described. Conditioned medium was dialysed (three changes of 4 l, dialysed overnight: M_r cutoff 3500; Spectrapor, Fisher Scientific, Pittsburgh, PA) against 10 mmol Tris l^{-1}, pH 7.4 and nondialysable radioactivity was determined. Aliquots containing 10^6 d.p.m. non-dialysable radioactivity were immunoprecipitated with 25 μl antiserum and subjected to SDS-PAGE and fluorography (4-month exposure). Immunoprecipitation of two to three times this volume of medium did not saturate the antibody (data not shown). Densitometry of fluorographs was used to quantitate relative amounts of immunoreactive RBP resulting from immunoprecipitation.

Experiment 3

Thirty-three crossbred neonatal gilts were randomly assigned at birth to receive either 10 000 iu retinyl palmitate day^{-1} (provided by Stuart Products Inc., Bedford, TX); 20 mg progesterone day^{-1}; 100 μg oestradiol day^{-1}; 1 or 0.1 mg tamoxifen day^{-1} or 0.5 ml vehicle day^{-1} (90% corn oil, 10% ethanol) from birth and continuing for 14 days. The dose of retinyl palmitate was 17 times greater than the requirement recommended by the National Research Council (1988) because of uncertainties about availability to uterine tissues. Blood samples (3–5 ml) were collected on days 5, 10 and 14 before daily injections. On day 14, gilts were weighed, killed and uteri collected. The broad ligament was trimmed and the uterus was weighed. For histological assessment, a 1 cm section of the uterine horn collected at the uterine bifurcation was fixed in 4% (w/v) paraformaldehyde PBS. Additional uterine tissue (200 mg) was cultured for 24 h in 0.1 × methionine MEM plus 25 μCi [^{35}S]methionine (1190 Ci $mmol^{-1}$, NEN Dupont, Wilmington, DE).

After incubation, [^{35}S]methionine radiolabelled culture medium was dialysed as previously described and nondialysable radioactivity was determined. Aliquots (1 ml), were then immunoprecipitated (25 μl antiserum or normal rabbit serum) and the immunoprecipitates were subjected to SDS-PAGE and fluorography (28-day exposure). The antiserum used in this experiment was raised against porcine RBP purified from allantoic fluid (Vallet, 1993). An additional 1 ml aliquot was lyophilized and subjected to two-dimensional PAGE and fluorography (28-day exposure) to examine secreted proteins other than immunoreactive RBP.

Assays for retinol-binding protein, retinol and progesterone

Plasma samples from control and retinyl palmitate-treated gilts were assayed for RBP (Vallet, 1994). Intra- and interassay coefficients of variation for the assay were 11.0 and 10.4%, respectively. The limit of detection of the assay was 0.46 ng.

Retinol in plasma was determined using a modification of the method of Selvaraj and Sushella (1970). Briefly, 0.5 ml of 1 mol KOH l^{-1} in 95% ethanol was added to 0.5 ml of serum and incubated at 60°C in capped tubes for 2 h. Samples were extracted with 3 ml xylene, frozen on dry ice and the xylene

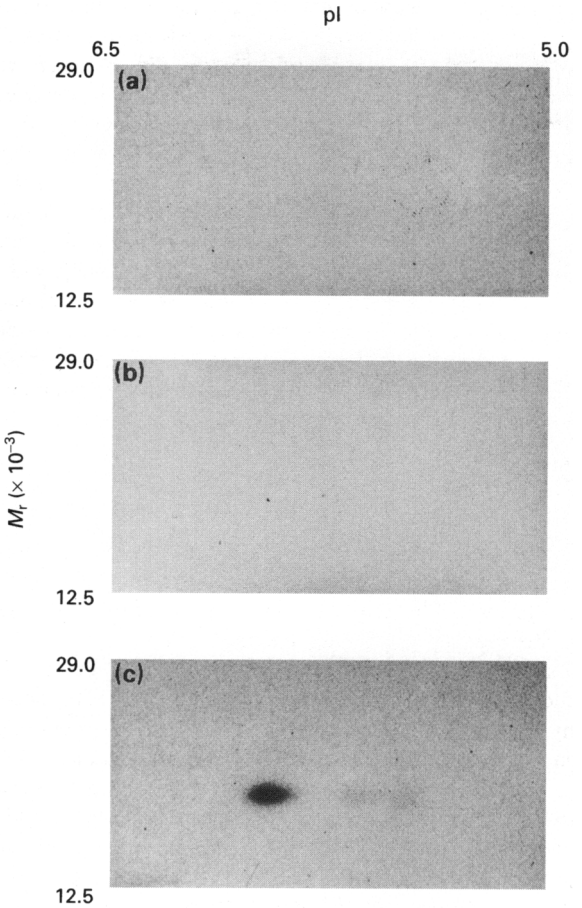

Fig. 1. Fluorographs (28-day exposure) of two-dimensional PAGE gels illustrating results of immunoprecipitation of [^{3}H]leucine-labelled uterine secreted proteins with either (a) normal rabbit serum (whole serum), (b) normal rabbit serum (rabbit gamma globulin) or (c) anti-human retinol-binding protein (RBP) antiserum (isolated IgG). Note spots in (c) indicating positive immunoprecipitation of immunoreactive RBP (M_r approximately 20 000; one major (pI approximately 5.6) and two minor (pI approximately 5.3 and 5.2) isoforms were observed).

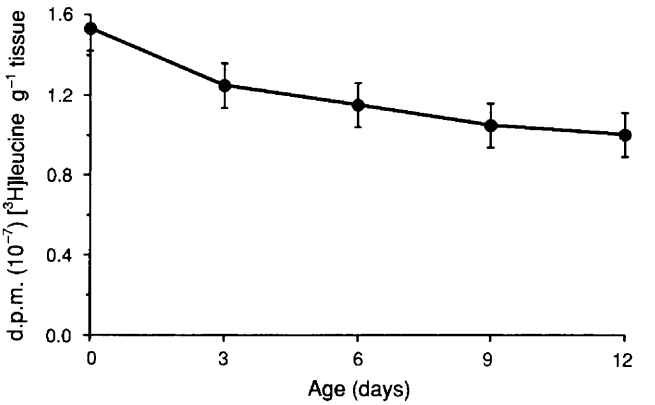

Fig. 2. Mean nondialysable radioactivity (d.p.m. 10^{-7} × [^{3}H]leucine g^{-1} tissue) in medium from cultures of uteri collected from 0-, 3-, 6-, 9- and 12-day-old gilts. A negative linear effect of day of age was detected ($P < 0.01$).

was collected. Retinol fluorescence in the xylene extracts was measured on a Perkin Elmer fluorometer at 330 nm excitation, 470 nm emission. Retinol concentrations were corrected for recovery (approximately 70%).

Plasma progesterone concentrations were determined by radioimmunoassay. The antibody used was prepared against progesterone-11–BSA (Cambridge Medical, Billerica, MA). The assay was validated by demonstrating quantitative recovery of exogenous progesterone added to plasma and parallelism of increasing volumes of plasma to the standard curve. The intra- and interassay coefficients of variation were 9.1 and 14.2%, respectively. The limit of detection of the assay was 0.15 pg.

Preparation and evaluation of histological sections

Fixed tissue was dehydrated, embedded in paraffin wax, sectioned (5 μm), mounted on slides and stained with haematoxylin and eosin. Two sections (at least 1 mm apart) from each uterus from each gilt were evaluated for total uterine cross-sectional, longitudinal myometrial, circular myometrial, endometrial, endometrial gland epithelial and luminal epithelial area to assess gland development. Morphometric analysis was performed using a Microcomp Integrated Image Analysis System (Southern Micro Instruments, Atlanta, GA), after calibration using a slide micrometer. The external border of the uterus, the border between the longitudinal and circular myometrium, the border between the myometrium and endometrium, the basal border of the luminal epithelium and the luminal border were encircled and the areas within these borders were calculated. Longitudinal myometrial area was defined as the total uterine cross-sectional area minus the area within the longitudinal–circular myometrial border. Similar calculations were made to obtain circular myometrial, endometrial and luminal epithelial area. Endometrial glands were defined as groups of epithelial cells that surrounded a closed lumen separated from the uterine lumen by interposed cells (Branham *et al.*, 1985b). To determine glandular epithelial area, the area within the lumen of each gland was determined, summed and subtracted from the sum of the areas of each gland.

Statistical analyses

Data for nondialysable radioactivity in culture medium from Expt 2 were subjected to analysis of variance, using a model that included the effect of day, and regression analysis, using day as a continuous independent variable. Data resulting from densitometry of the 20 000 M_r immunoreactive RBP band (area under the curve) were log transformed and subjected to analysis of variance using a model that included the effects of gel (four samples were run on each gel for a total of five gels) and day. This analysis is reported because gels were loaded on the basis of similar nondialysable radioactivity, a procedure used by Spencer *et al.* (1992). However, because the amount of nondialysable radioactivity decreased linearly with age of the gilt, sample loading based on nondialysable radioactivity may not fully compensate for differences in tissue masses. Therefore, in a separate analysis, these data were expressed as relative

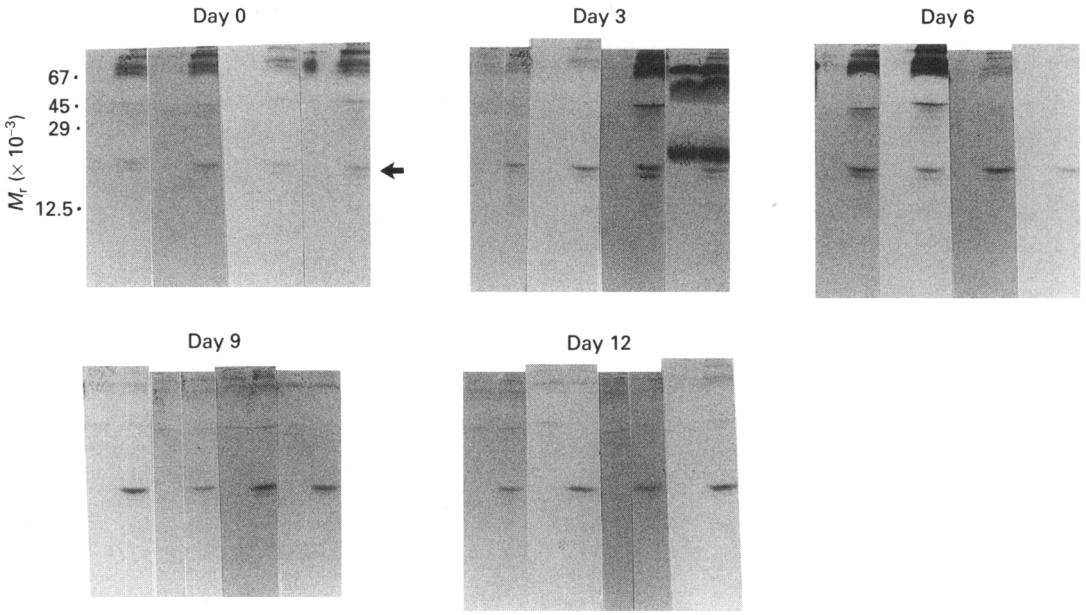

Fig. 3. Fluorographs (4-month exposure) resulting from immunoprecipitation of [^{3}H]leucine-labelled proteins secreted into culture medium by uterine tissue from 0-, 3-, 6-, 9- or 12-day-old gilts ($n = 4$ at each age). Fluorographs are arranged in pairs depicting results obtained from individual gilts. For each pair at each age, proteins immunoprecipitated using rabbit gamma globulin (negative control) are in the first lane and proteins immunoprecipitated with anti-retinol-binding protein (RBP) are in the second lane. The arrow indicates the position of immunoreactive RBP bands. Note the presence of two bands of immunoreactive RBP (approximately 20 000 and 19 000 M_r) on days 0 to 6 and the absence of the lower molecular weight band by day 12. The other bands represent proteins that are precipitated by anti-human RBP and normal rabbit serum (not shown), but that are not precipitated by rabbit gamma globulin.

units per milligramme tissue to correct for tissue masses in each culture, and then subjected to analysis of variance using a model that included the effects of gel and day. In both analyses, the following set of orthogonal contrasts was performed: day 9 versus day 12; day 6 versus days 9 and 12 combined; day 3 versus days 6, 9 and 12 combined; and day 0 versus days 3, 6, 9 and 12 combined. Densitometry data for the 19 000 M_r RBP band were analysed as described above, except that the following set of orthogonal contrasts was used: day 3 versus day 6; day 1 versus days 3 and 6 combined; day 9 versus day 12; and days 0, 3 and 6 combined versus days 9 and 12 combined.

For Expt 3, plasma RBP, retinol and progesterone were analysed by analysis of variance, using a model that included effects of treatment, gilt within treatment, day of age and day of age by treatment interaction. Simple correlations were calculated between RBP secreted in culture, incorporation of [^{35}S]methionine into nondialysable radioactivity, uterine area, longitudinal myometrial area, circular myometrial area, endometrial area, glandular epithelial area and luminal epithelial area. Areas of uterine components were analysed with and without log transformation using a model that included effects of treatment and gilt within treatment. The untransformed error variance and gilt within treatment variance were used to calculate repeatability estimates of these measurements. Log-transformed data and the following contrasts were used to examine treatment effects in detail: control versus progesterone-treated groups (effect of progesterone); control versus retinyl palmitate-treated group (effect of retinyl palmitate); control versus 0.1 mg tamoxifen day^{-1} treated group (effect of low tamoxifen dose); oestradiol-treated group versus 1 mg tamoxifen day^{-1} treated group (comparison of the two potentially oestrogenic treatments) and control, progesterone, retinyl palmitate and 0.1 mg tamoxifen day^{-1} treated groups combined versus oestradiol and 1 mg tamoxifen day^{-1} treated groups combined (comparison of potentially non-oestrogenic treatments with oestrogenic treatments). The first three contrasts are not orthogonal but each is orthogonal with the last two contrasts; the last two contrasts are orthogonal with each other. Body mass on day 14, uterine mass, RBP secreted in culture and incorporation of [^{35}S]methionine into nondialysable macromolecules were analysed by analysis of variance using a model that included the effect of treatment. The contrasts described above were used to define treatment differences. RBP secreted in culture was log transformed before analysis.

Results

Experiment 1

The anti-human RBP antiserum specifically immunoprecipitated one major (pI = 5.6) and two minor (pI = 5.3, 5.2) isoforms of a protein with M_r of approximately 20 000 (Fig. 1).

Experiment 2

Nondialysable radioactivity per gram of uterine tissue cultured decreased linearly ($P < 0.01$) with increase in age (Fig. 2).

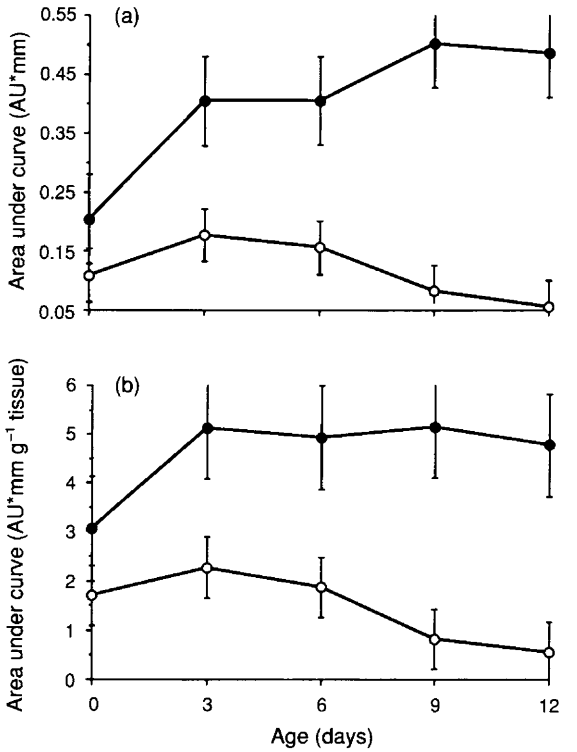

Fig. 4. Means for densitometric measurements (area under the curve) for both the 20 000 and 19 000 M_r bands from immunoprecipitation analysis of uterine proteins from neonate gilts using anti-human retinol-binding protein (RBP) antiserum (see Fig. 3). (a) Area under the curve and (b) area under the curve corrected for cultured tissue mass are illustrated. Secretion of (●) the 20 000 M_r protein immunoreactive (RBP) increased ($P < 0.01$ before and $P = 0.07$ after correction for tissue mass) from day 0 to day 3 and then did not change during days 3–12. Secretion of (○) the 19 000 M_r protein (possible immunoreactive RBP) did not change from days 0–6, decreased ($P < 0.05$ before and after correction for tissue cultured) from day 6 to day 9 and remained low on day 12.

Visual inspection of the fluorographs indicated that uterine secretion of immunoreactive RBP increased as early as day 3, and that uteri of 0-, 3- and 6-day-old gilts secreted two M_r variants of immunoreactive RBP with approximate M_r of 20 000 and 19 000 (Fig. 3). Secretion of the lower M_r variant decreased on days 9 and 12. These results were confirmed by densitometry (Fig. 4). Orthogonal contrasts comparing the secretion of the 20 000 M_r immunoreactive RBP variant indicated that secretion of this variant increased ($P < 0.01$ before and $P = 0.07$ after correction for tissue mass in culture) from day 0 to day 3 but did not differ from days 3–12. Secretion of the 19 000 M_r variant did not differ from day 0 to day 6, significantly decreased ($P < 0.05$ before and after correction for tissue mass cultured) from day 6 to day 9 and remained low on day 12.

Experiment 3

Plasma RBP increased (main effect of treatment, $P = 0.06$) in retinyl palmitate-treated gilts (least squares means ± SEM was 49.5 ± 3.6 μg ml^{-1}) compared with controls (38.6 ± 4.0 μg ml^{-1}) as did plasma retinol (5430 ± 208 compared with 256 ± 232 ng ml^{-1}, respectively; $P < 0.01$). Plasma progesterone increased (main effect of treatment, $P < 0.01$) in progesterone-treated gilts (12.1 ± 0.50 ng ml^{-1}) compared with controls (0.18 ± 0.50 ng ml^{-1}). No effect of day, or treatment by day interaction, was detected for plasma RBP, retinol or progesterone.

Repeatability of histomorphometric measurements of total uterine cross-sectional, longitudinal myometrial, circular myometrial, endometrial, glandular epithelial and luminal epithelial areas were 0.99, 0.96, 0.98, 0.99, 0.96 and 0.96, respectively. Duplicate measurements are therefore more than sufficient to measure treatment effects on these uterine components.

Least squares means for body mass, uterine mass, RBP secreted in culture, incorporation of [^{35}S]methionine into nondialysable macromolecules and total uterine cross-sectional, longitudinal myometrial, circular myometrial, endometrial, glandular epithelial and luminal epithelial areas are summarized (Table 1). No treatment effects were detected for body mass at 14 days of age. Uterine masses of gilts treated with oestradiol or 1 mg tamoxifen day^{-1} did not differ but were greater ($P < 0.01$) than masses from the other treatment groups combined. Treatment with retinyl palmitate, progesterone or 0.1 mg tamoxifen day^{-1} had no effect on uterine mass. Progesterone treatment decreased ($P < 0.05$) uterine secretion of immunoreactive RBP compared with controls. Secretion of immunoreactive RBP by gilts treated with oestradiol or 1 mg tamoxifen day^{-1} did not differ but was greater ($P < 0.01$) than the other treatment groups combined. Treatment with retinyl palmitate or 0.1 mg tamoxifen day^{-1} had no effect on immunoreactive RBP secretion. None of the treatments affected uterine incorporation of [^{35}S]methionine into nondialysable macromolecules. Photomicrographs of representative cross-sections of uteri from the different treatment groups are shown (Fig. 5).

Total uterine cross-sectional, longitudinal myometrial, circular myometrial, endometrial, glandular epithelial and luminal epithelial areas were increased ($P < 0.01$) by treatment with oestradiol and 1 mg tamoxifen day^{-1} compared with the other treatment groups combined. However, oestradiol increased total uterine cross-sectional ($P < 0.05$), longitudinal myometrial ($P < 0.01$), circular myometrial ($P < 0.05$) and endometrial ($P < 0.05$) areas significantly more than treatment with 1 mg tamoxifen day^{-1}. The effects of oestradiol and tamoxifen (1 mg day^{-1}) did not differ for glandular and luminal epithelial area. Tamoxifen (0.1 mg day^{-1}; $P < 0.05$) and retinyl palmitate ($P < 0.05$) increased glandular epithelial area but had no effect on other uterine components. Progesterone treatment had no effect on any uterine component measured.

Simple correlations between body mass at 14 days of age, uterine mass, uterine secretion of immunoreactive RBP, incorporation of [^{35}S]methionine into nondialysable macromolecules, and total uterine cross-sectional, longitudinal myometrial, circular myometrial, endometrial, glandular epithelial and luminal epithelial areas are summarized (Table 2). Body mass (r ranging from 0.36 to 0.53) and uterine mass (r ranging from 0.66 to 0.80) were moderately correlated with the uterine components. Uterine immunoreactive RBP was also correlated with the uterine components (r ranging from 0.38 to 0.68); the highest correlation was with glandular epithelial

Table 1. Least squares means (± SEM) for body mass at 14 days of age, uterine mass, uterine immunoreactive RBP secretion, uterine incorporation of [^{35}S]methionine into nondialysable macromolecules, and total uterine cross-sectional, longitudinal myometrial, circular myometrial, endometrial, glandular epithelial and luminal epithelial areas for gilts receiving different treatments

Characteristic	Treatment					
	Control	Retinyl palmitate (10 000 iu day^{-1})	Progesterone (20 mg day^{-1})	Oestradiol (100 mg day^{-1})	Tamoxifen (1 mg day^{-1})	Tamoxifen (0.1 mg day^{-1})
Body mass (kg)	3.50 ± 0.32 (5)[a]	3.96 ± 0.29 (6)	3.40 ± 0.32 (5)	3.76 ± 0.29 (6)	4.06 ± 0.32 (5)	3.70 ± 0.29 (6)
Uterine mass (g)[b]	0.43 ± 0.18 (5)	0.58 ± 0.16 (6)	0.46 ± 0.18 (5)	1.31 ± 0.16 (6)	1.37 ± 0.18 (5)	0.60 ± 0.16 (6)
Immunoreactive RBP (relative units)[bc]	0.71 ± 0.30 (3)	0.40 ± 0.21 (5)	0.31 ± 0.21 (5)	1.23 ± 0.19 (6)	1.04 ± 0.24 (4)	0.49 ± 0.21 (5)
[^{35}S]methionine in nondialysable macromolecules (c.p.m. × 10^{-6} g^{-1} tissue)	3.85 ± 0.37 (5)	3.83 ± 0.33 (6)	3.71 ± 0.37 (5)	3.65 ± 0.33 (6)	3.94 ± 0.37 (5)	3.82 ± 0.33 (6)
Total uterine cross-sectional area (mm^2)[bd]	2.3 ± 0.7 (5)	3.1 ± 0.6 (6)	2.6 ± 0.7 (5)	6.8 ± 0.6 (6)	4.2 ± 0.7 (5)	3.5 ± 0.6 (6)
Longitudinal myometrial area (mm^2)[be]	0.23 ± 0.11 (5)	0.31 ± 0.09 (6)	0.25 ± 0.11 (5)	0.91 ± 0.09 (6)	0.44 ± 0.11 (5)	0.31 ± 0.09 (6)
Circular myometrial area (mm^2)[bd]	1.03 ± 0.26 (5)	1.34 ± 0.23 (6)	1.13 ± 0.26 (5)	2.59 ± 0.23 (6)	1.70 ± 0.26 (5)	1.45 ± 0.23 (6)
Endometrial area (mm^2)[bd]	0.95 ± 0.36 (5)	1.37 ± 0.32 (6)	1.05 ± 0.36 (5)	3.06 ± 0.32 (6)	1.91 ± 0.36 (5)	1.59 ± 0.32 (6)
Glandular epithelial area (mm^2)[bfg]	0.039 ± 0.065 (5)	0.092 ± 0.058 (6)	0.027 ± 0.065 (5)	0.427 ± 0.058 (6)	0.240 ± 0.065 (5)	0.107 ± 0.058 (6)
Luminal epithelial area (mm^2)[b]	0.089 ± 0.029 (5)	0.096 ± 0.025 (6)	0.100 ± 0.029 (5)	0.200 ± 0.025 (6)	0.125 ± 0.029 (5)	0.122 ± 0.025 (6)

[a]Number of observations is in parentheses.
[b]Oestradiol and 1 mg tamoxifen day^{-1} treatment combined were significantly different ($P < 0.01$) from control, retinyl palmitate, progesterone and 0.1 mg tamoxifen day^{-1} treatment combined.
[c]Progesterone treatment was significantly different from control ($P < 0.05$).
[d]Oestradiol was significantly different from 1 mg tamoxifen day^{-1} treatment ($P < 0.05$).
[e]Oestradiol was significantly different from 1 mg tamoxifen day^{-1} treatment ($P < 0.01$).
[f]Retinyl palmitate treatment was significantly different from control treatment ($P < 0.05$).
[g]0.1 mg tamoxifen day^{-1} treatment was significantly different from control treatment ($P < 0.05$).

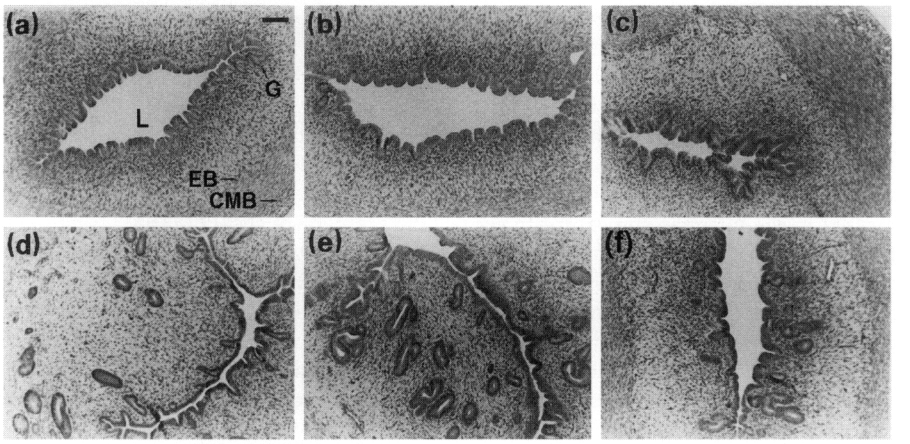

Fig. 5. Photomicrographs of representative cross-sections of uteri from neonatal gilts treated with (a) corn oil, (b) retinyl palmitate (10 000 iu day $^{-1}$), (c) progesterone (20 mg day $^{-1}$), (d) oestradiol (100 µg day $^{-1}$), tamoxifen (e) 1 mg day $^{-1}$, (f) 0.1 mg day $^{-1}$. The uterine lumen (L), an endometrial gland (G) and the endometrial (EB) and circular myometrial borders (CMB) are indicated in (a). Quantitation of areas of each component in cross-sections of the uterus are summarized in Table 1. Scale bar represents 100 µm.

Table 2. Simple correlations between body mass at 14 days of age (BMT), uterine mass (UMT) uterine immunoreactive retinol-binding protein secretion (irRBP), uterine incorporation of [^{35}S]methionine into nondialysable macromolecules (UINDM), and total uterine cross-sectional (TUCA), longitudinal myometrial (LMA), circular myometrial (CMA), endometrial (EA), glandular epithelial (GEA) and luminal epithelial (LEA) areas for gilts in Experiment 3

	UMT	irRBP	UINDM	TUCA	LMA	CMA	EA	GEA	LEA
BMT[a]	0.53[c]	0.02	0.09	0.40[b]	0.42[b]	0.36[b]	0.40[b]	0.37[b]	0.53[c]
UMT		0.53[c]	− 0.03	0.74[c]	0.67[c]	0.66[c]	0.78[c]	0.80[c]	0.73[c]
irRBP			0.22	0.56[c]	0.47[c]	0.51[c]	0.60[c]	0.68[c]	0.38[b]
UINDM				− 0.02	− 0.01	− 0.07	0	− 0.04	0.08
TUCA					0.93[c]	0.97[c]	0.98[c]	0.92[c]	0.88[c]
LMA						0.89[c]	0.86[c]	0.85[c]	0.86[c]
CMA							0.92[c]	0.83[c]	0.81[c]
EA								0.94[c]	0.86[c]
GEA									0.75[c]

[a]Number of observations for each correlation was 28 for correlations with irRBP and 33 for all others.
[b]Correlation is significant ($P < 0.05$).
[c]Correlation is significant ($P < 0.01$).

area. Uterine components were all highly correlated with each other (r ranging from 0.75 to 0.98). Uterine incorporation of [^{35}S]methionine into nondialysable macromolecules was not correlated with any other trait measured.

Discussion

Results of the work presented here indicate that a protein that crossreacts with anti-human RBP antiserum is synthesized and secreted by the uteri of neonatal gilts; secretion of immunoreactive RBP increases coincident with initiation of uterine gland development; treatment with retinyl palmitate increases endometrial gland epithelial area without influencing other uterine components; and oestradiol and tamoxifen treatments stimulate endometrial gland development and uterine immunoreactive RBP secretion. In contrast to the effect of progesterone on endometrial RBP secretion in mature gilts (Adams *et al.*, 1981), secretion of immunoreactive RBP by uterine tissues of neonatal gilts was inhibited by progesterone treatment. The 20 000 M_r form of immunoreactive RBP identified here has the same M_r and similar pI as serum and uterine RBP from mature gilts (Rask, 1974; Clawitter *et al.*, 1990; Harney *et al.*, 1990). Together, these results suggest that the protein is RBP, and that RBP and retinol play a role in the development of uterine glands in neonates. However, owing to contamination of the cultures with RBP from serum, it was not possible using amino-terminal sequencing or retinol-binding studies to ascertain whether this protein was RBP.

The increase in immunoreactive RBP secretion that occurs coincident with the initiation of uterine gland development

suggests that increased immunoreactive RBP secretion either participates in, or results from, gland development. However, it is possible that the coincident increase is unrelated to the process of gland development. Neonatal gilts were treated with retinyl palmitate to observe its effect on gland epithelial area in uterine cross-sections. Although the dosage given was higher than physiological concentrations, it is difficult to assess whether the dosage resulted in the pharmacological treatment of gilts with retinol, because the rate of conversion of retinyl palmitate to retinol by uterine tissues and their ability to take up retinol in this form are not known. Furthermore, tissues (such as the liver) may metabolize retinyl palmitate to retinol and secrete the retinol bound to RBP which would then be available to other tissues. Results indicated that while retinol in the plasma (a measure of retinyl palmitate and other retinoids) was high, RBP was increased by 28%, which is a physiological increase. However, the observation that treatment with retinyl palmitate specifically stimulated gland epithelial area suggests that retinol participates in the process of gland development. Although it was not possible to distinguish between hyperplastic and hypertrophic growth in this experiment, either type of growth may be involved in normal uterine gland development. As RBP is the normal carrier of retinol, immunoreactive RBP may participate in gland development. Although retinyl palmitate did not stimulate uterine secretion of immunoreactive RBP, it did stimulate plasma RBP concentrations. It seems plausible that uterine secretion of immunoreactive RBP is influenced by plasma RBP or retinol concentrations. Thus exogenous retinol could stimulate gland development without coincident increased stimulation of uterine immunoreactive RBP.

The effect of progesterone treatment on total protein and uterine secretion of immunoreactive RBP differs in neonatal gilts from that in mature gilts. Concentrations of progesterone in the plasma of the progesterone-treated neonatal gilts were similar to those that occur during pregnancy (Robertson and King, 1974) and the dosage used was similar to that used to induce secretion of endometrial protein (Knight *et al.*, 1974) and RBP (Adams *et al.*, 1981) in mature gilts. Decreased secretion of immunoreactive RBP suggests that the neonatal uterus has functional progesterone receptors. Differences in progesterone receptor concentrations, distribution or function might explain the difference observed in neonatal and mature uterine tissues.

Treatment of neonatal ewes with the synthetic progestin norgestomet inhibited uterine gland development (Bartol *et al.*, 1988). Treatment of neonatal mice with 40 mg progesterone kg^{-1} inhibited uterine epithelial [^{3}H]thymidine uptake and the mitotic index (Bigsby and Cunha, 1985). These findings suggest that the withdrawal of maternal progesterone that occurs at birth influences uterine gland development. However, the results reported here do not support this contention for gilts.

Results from this study indicate that tamoxifen acts as an oestrogen agonist in pigs (as previously reported by Lin and Buttle, 1991), and that tamoxifen stimulated uterine gland development while having little influence on other components of the uterus. These results are in contrast to those found in rats by Branham *et al.* (1985a). Thus, the response of different species to oestrogen agonist or antagonist compounds differ and care is required when predicting the response to a particular oestrogenic or anti-oestrogenic compound.

Two M_r forms of immunoreactive RBP were immunoprecipitated from medium in which neonatal uterine tissues were cultured. However, the identification of the 19 000 M_r band as RBP is inconclusive because many bands, in addition to immunoreactive RBP, appear to be specifically immunoprecipitated. There appears to be only a single gene for RBP in pigs (Stallings-Mann *et al.*, 1993). Thus, a possible explanation for the presence of the 19 000 M_r band is proteolytic cleavage of the 20 000 M_r form; proteolytic cleavage of RBP could release retinol to target tissues. Alternatively, the 19 000 M_r protein could be nonspecifically bound by the antiserum. Regardless of the correct explanation, the observation that the amount of this protein changes coincident with initiation of uterine gland development makes it of interest and the origin of this band should be investigated.

Uterine mass and the areas of all components of uterine structure correlated with body mass, suggesting that, during the period examined, nutrition of neonatal gilts influences uterine growth and development. Nutrition in piglets depends on milk supply, which is influenced by maternal milk production and competition among littermates. The influence of differences in neonatal nutrition on later uterine function requires further investigation.

In conclusion, this study demonstrates that a protein with immunochemical crossreactivity with RBP (immunoreactive RBP) is secreted by porcine uteri in association with the initiation of uterine gland development. Treatment with oestradiol and 1 mg tamoxifen day^{-1} stimulated both uterine gland development and immunoreactive RBP secretion. Gland epithelial area had the highest correlation with uterine immunoreactive RBP secretion of the uterine wall components. Retinyl palmitate treatment stimulated uterine gland epithelial area without affecting other uterine components. These results suggest an association between uterine gland development and uterine RBP secretion. Further studies are necessary to elucidate the role of retinol and RBP in neonatal uterine gland development.

Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the same by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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